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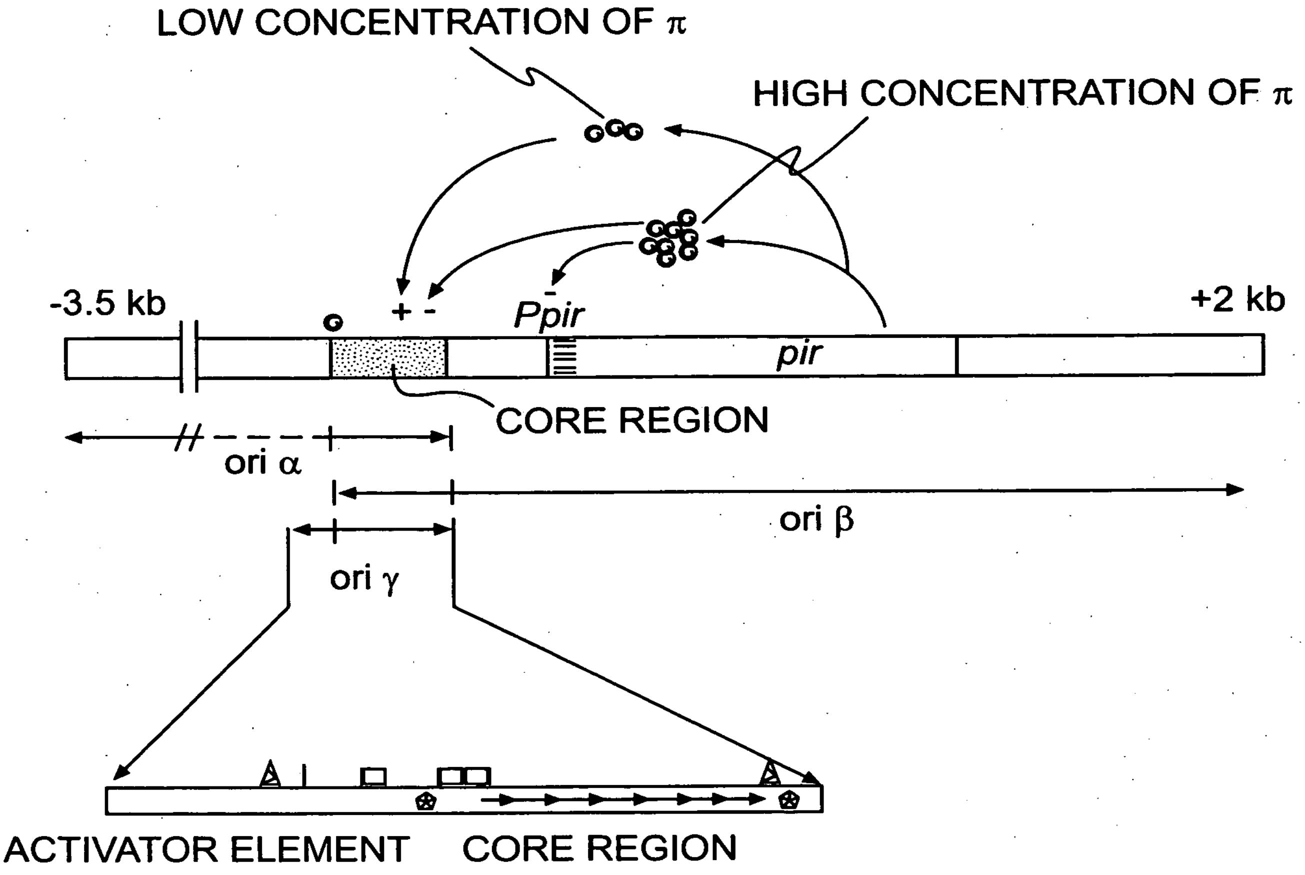


KEY:

- ⬟ BINDING SITE FOR IHF FACTOR
- □ DnaA CONSENSUS SEQUENCES
- △ DnaA BOX NO. 1
- → ITERON (22 bp SEQUENCE)
- ⊙ π PROTEIN

FIG. 1

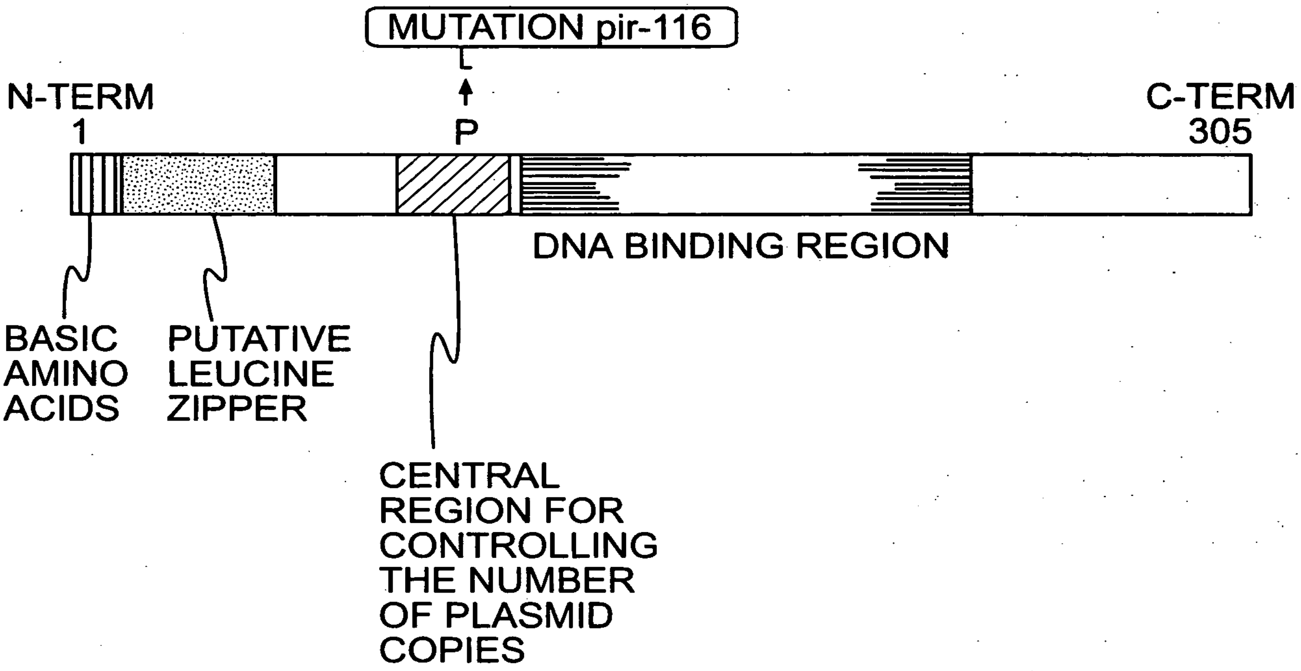

MUTATION pir-116
L
P
N-TERM
1
C-TERM
305
DNA BINDING REGION
BASIC AMINO ACIDS
PUTATIVE LEUCINE ZIPPER
CENTRAL REGION FOR CONTROLLING THE NUMBER OF PLASMID COPIES

FIG. 2

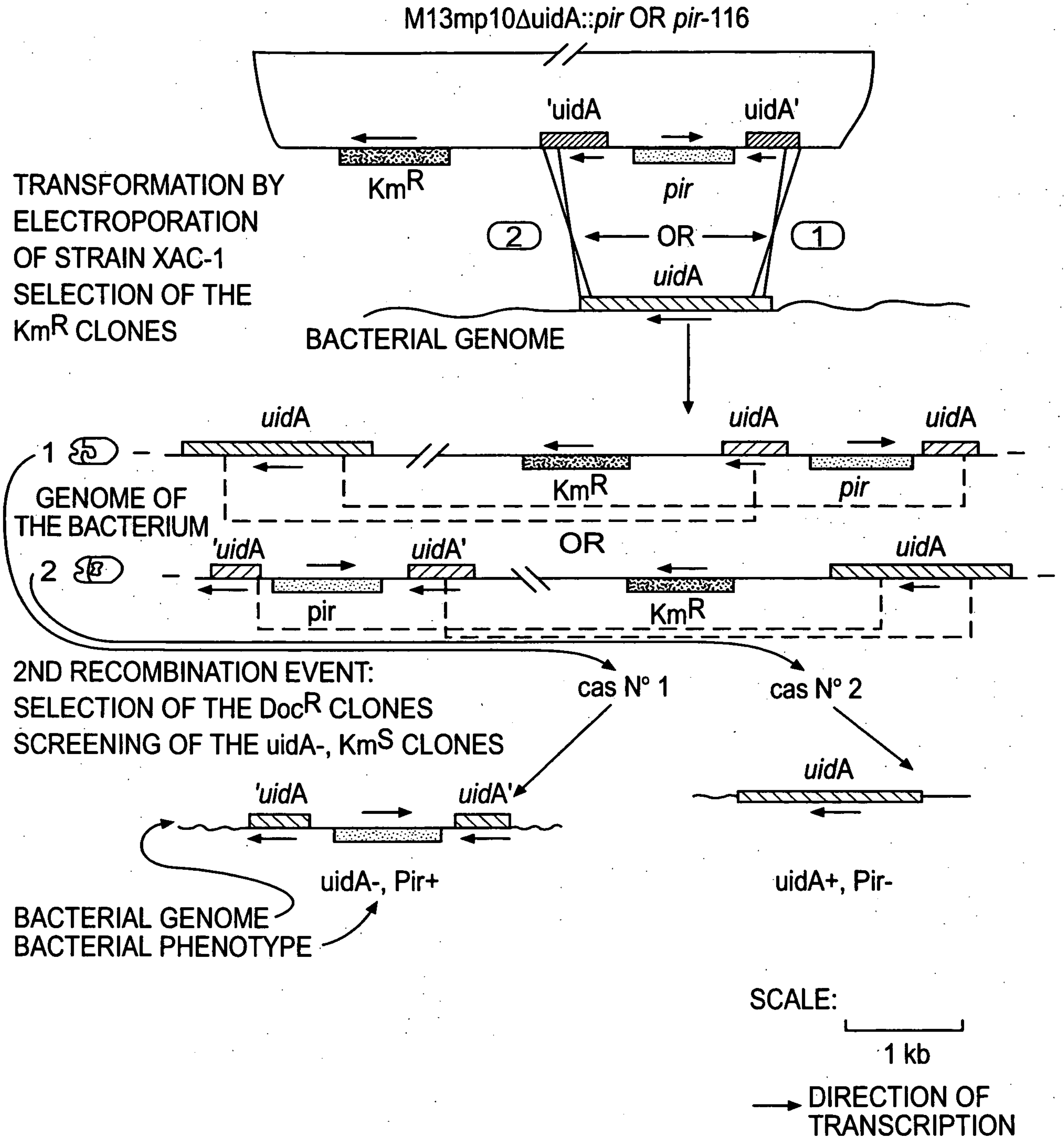

M13mp10ΔuidA::pir OR pir-116
'uidA
uidA'
KmR
pir
TRANSFORMATION BY ELECTROPORATION OF STRAIN XAC-1 SELECTION OF THE KmR CLONES
2
OR
1
uidA
BACTERIAL GENOME
uidA
uidA
uidA
1
KmR
pir
GENOME OF THE BACTERIUM
OR
'uidA
uidA'
uidA
2
pir
KmR
2ND RECOMBINATION EVENT: SELECTION OF THE DocR CLONES SCREENING OF THE uidA-, KmS CLONES
cas N° 1
cas N° 2
'uidA
uidA'
uidA
uidA-, Pir+
uidA+, Pir-
BACTERIAL GENOME
BACTERIAL PHENOTYPE
SCALE:
1 kb
DIRECTION OF TRANSCRIPTION

FIG. 3

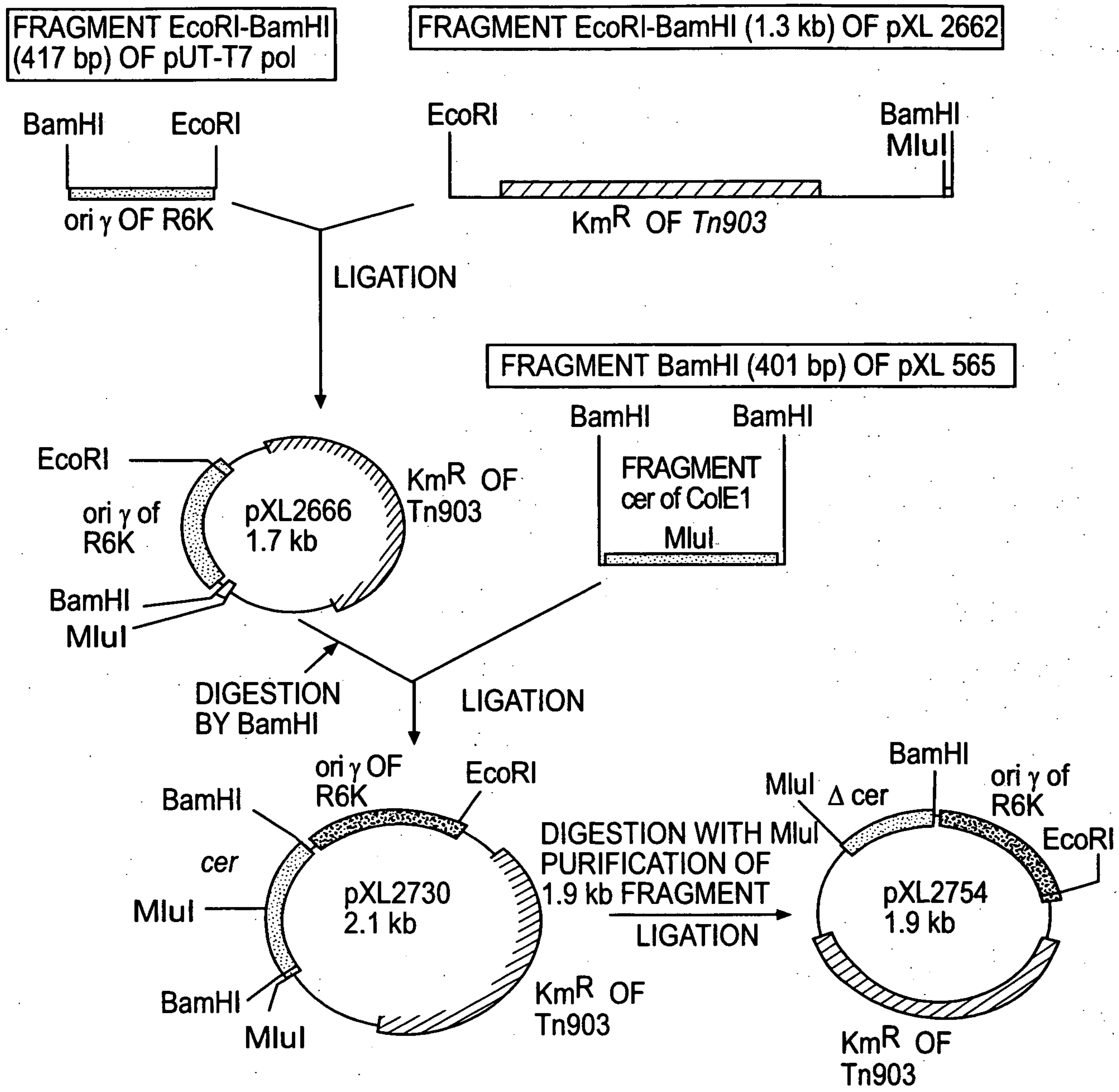

FRAGMENT EcoRI-BamHI (417 bp) OF pUT-T7 pol
BamHI
EcoRI
ori γ OF R6K
FRAGMENT EcoRI-BamHI (1.3 kb) OF pXL 2662
EcoRI
BamHI
MluI
Km^R OF Tn903
LIGATION
FRAGMENT BamHI (401 bp) OF pXL 565
BamHI
BamHI
FRAGMENT cer of ColE1
MluI
EcoRI
ori γ of R6K
pXL2666 1.7 kb
Km^R OF Tn903
BamHI
MluI
DIGESTION BY BamHI
LIGATION
ori γ OF R6K
EcoRI
BamHI
cer
MluI
pXL2730 2.1 kb
BamHI
MluI
Km^R OF Tn903
DIGESTION WITH MluI
PURIFICATION OF 1.9 kb FRAGMENT
LIGATION
BamHI
MluI
Δ cer
ori γ of R6K
EcoRI
pXL2754 1.9 kb
Km^R OF Tn903

FIG. 4

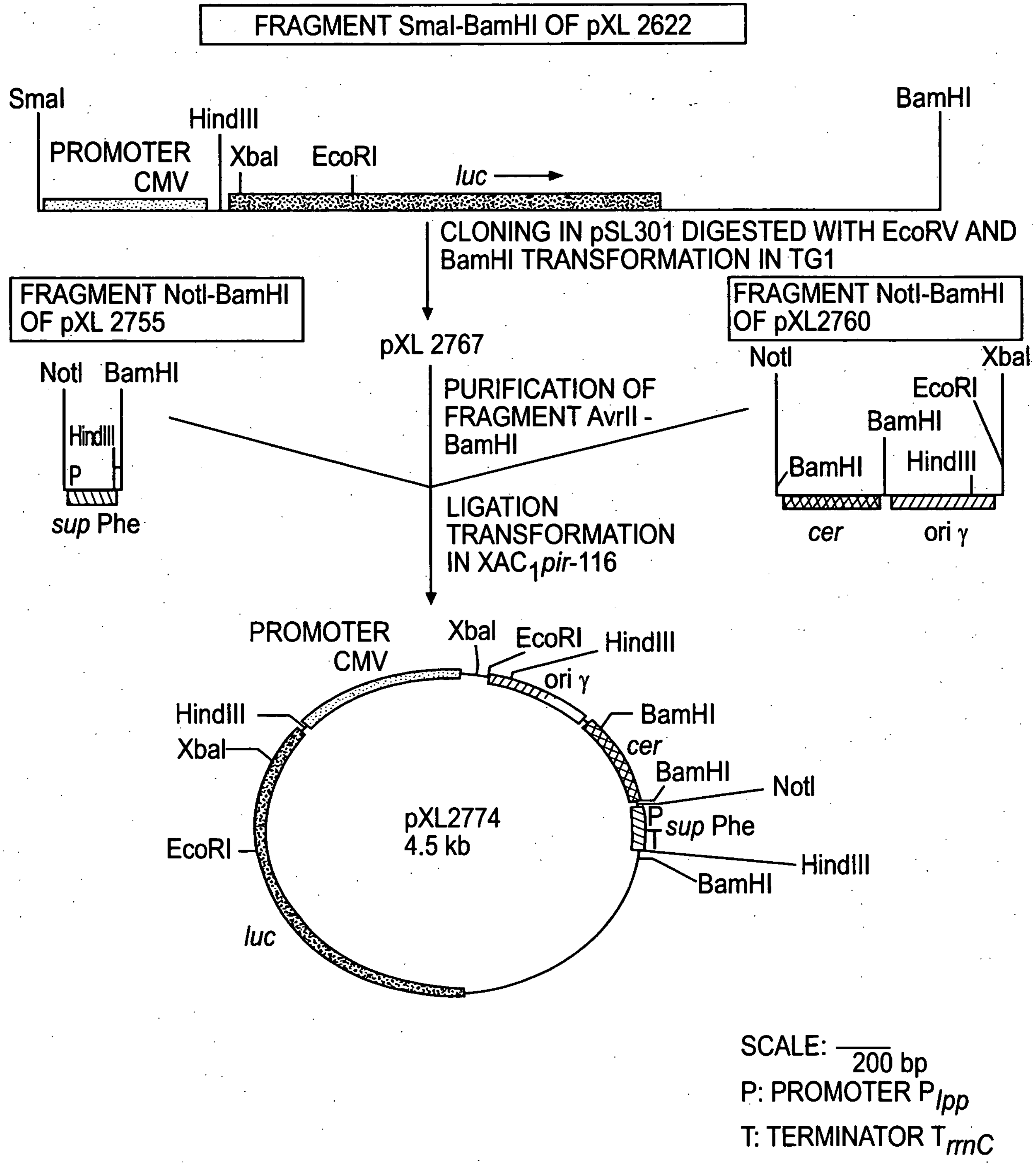

FRAGMENT SmaI-BamHI OF pXL 2622
SmaI
HindIII
BamHI
PROMOTER CMV
XbaI
EcoRI
luc
CLONING IN pSL301 DIGESTED WITH EcoRV AND BamHI TRANSFORMATION IN TG1
FRAGMENT NotI-BamHI OF pXL 2755
pXL 2767
FRAGMENT NotI-BamHI OF pXL2760
NotI
BamHI
HindIII
P
sup Phe
PURIFICATION OF FRAGMENT AvrII - BamHI
NotI
XbaI
EcoRI
BamHI
BamHI
HindIII
cer
ori γ
LIGATION TRANSFORMATION IN XAC$_1$*pir*-116
PROMOTER CMV
XbaI
EcoRI
HindIII
ori γ
HindIII
XbaI
BamHI
cer
BamHI
NotI
P
T
sup Phe
pXL2774
4.5 kb
EcoRI
HindIII
BamHI
luc
SCALE: 200 bp
P: PROMOTER P_{lpp}
T: TERMINATOR T_{rrnC}

FIG. 5

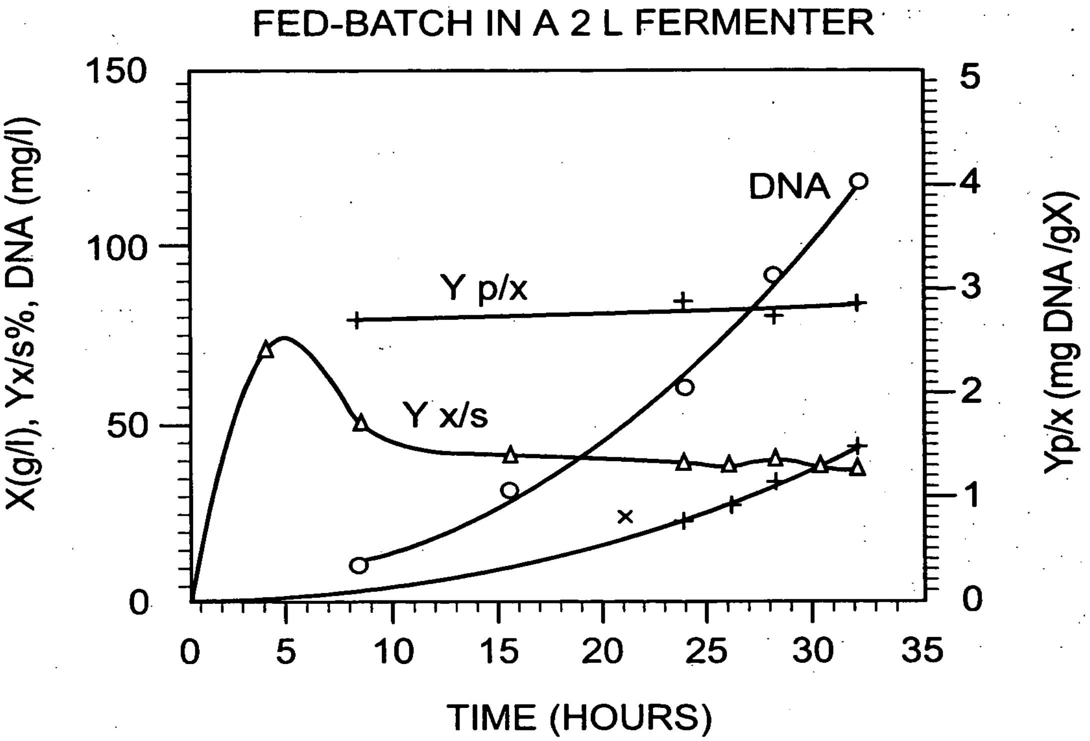

FED-BATCH IN A 2 L FERMENTER
X(g/l), Yx/s%, DNA (mg/l)
150
100
50
0
Yp/x (mg DNA /gX)
5
4
3
2
1
0
0
5
10
15
20
25
30
35
TIME (HOURS)
DNA
Y p/x
Y x/s

FIG. 6

FED-BATCH IN A 800 L FERMENTER

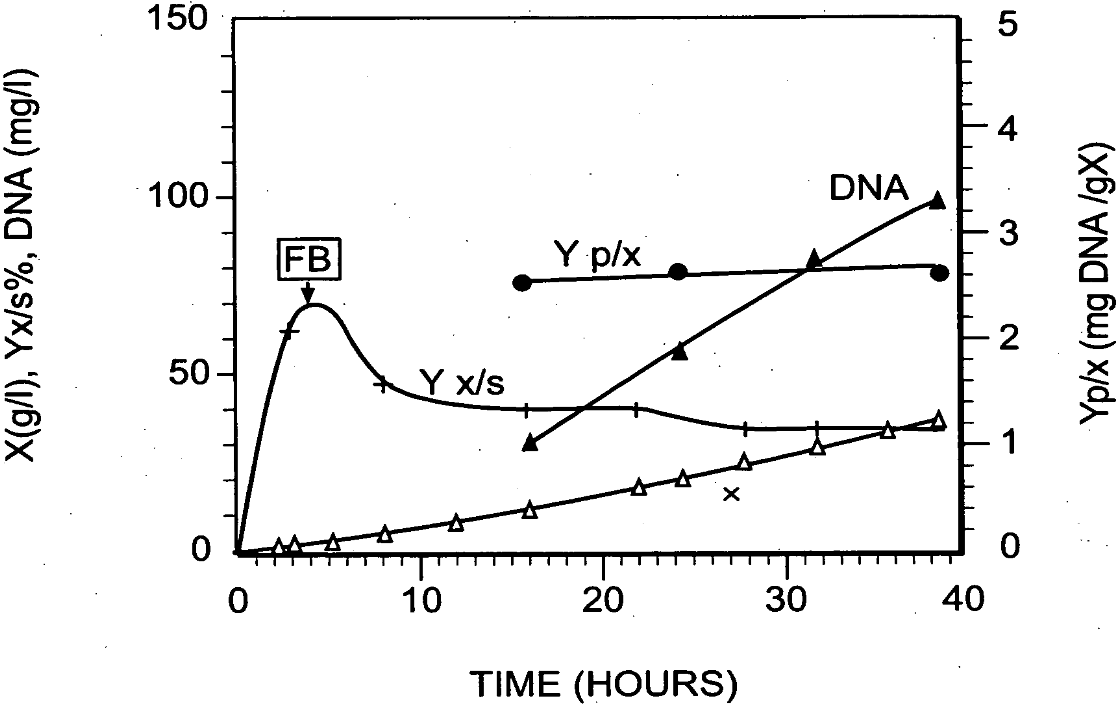


FIG. 7

CONSTRUCTION OF THE EXPRESSION VECTOR pXL3056

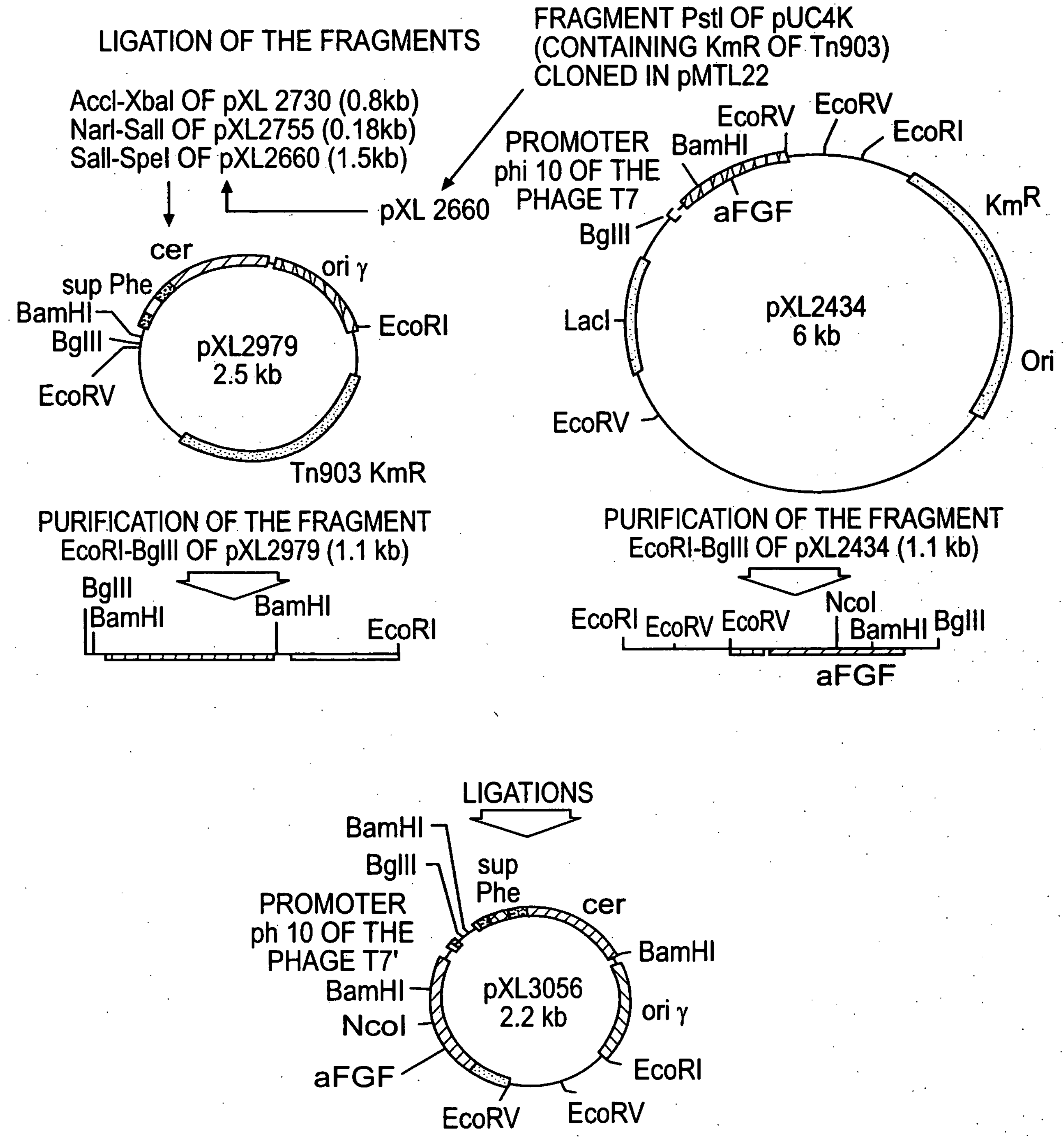


FIG. 8

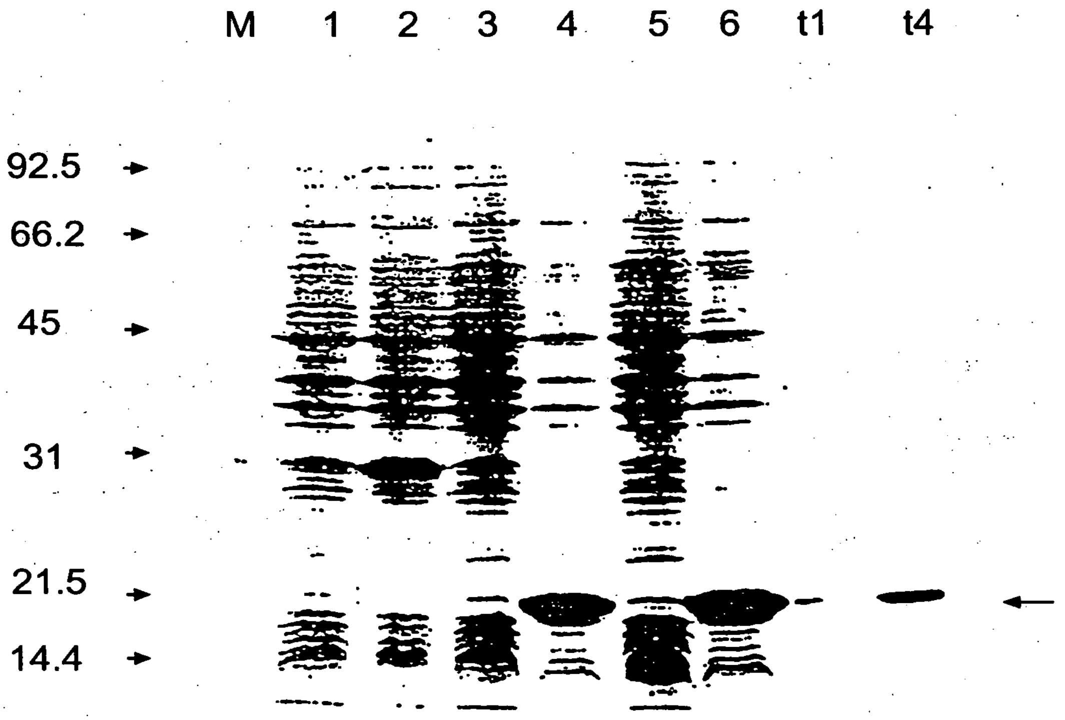


FIG. 9

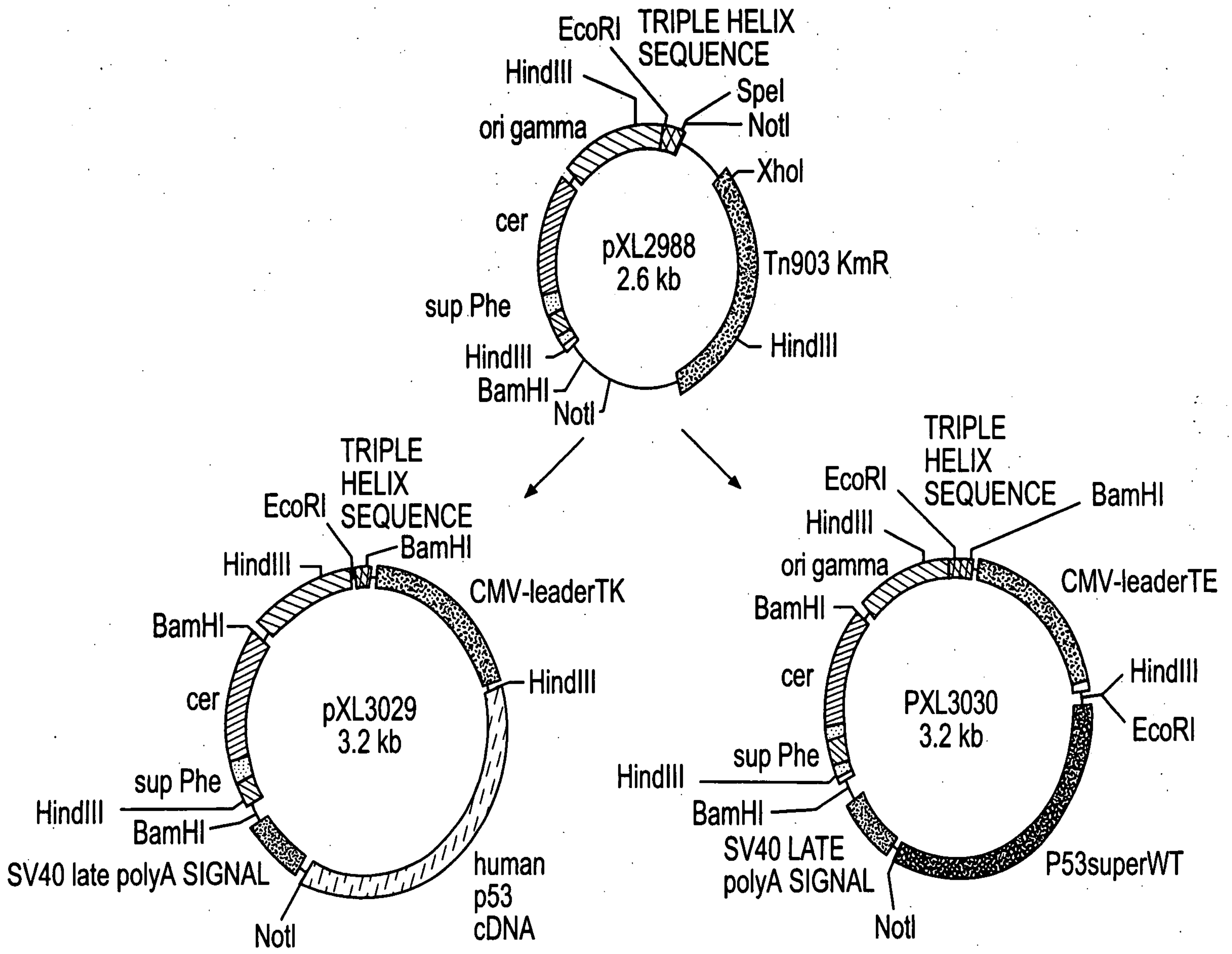

EcoRI
TRIPLE HELIX SEQUENCE
HindIII
SpeI
NotI
ori gamma
XhoI
cer
pXL2988
2.6 kb
Tn903 KmR
sup Phe
HindIII
HindIII
BamHI
NotI
TRIPLE HELIX SEQUENCE
EcoRI
BamHI
HindIII
CMV-leaderTK
BamHI
pXL3029
3.2 kb
HindIII
cer
sup Phe
HindIII
BamHI
SV40 late polyA SIGNAL
NotI
human p53 cDNA
TRIPLE HELIX SEQUENCE
EcoRI
BamHI
HindIII
ori gamma
CMV-leaderTE
BamHI
PXL3030
3.2 kb
HindIII
EcoRI
cer
sup Phe
HindIII
BamHI
SV40 LATE polyA SIGNAL
P53superWT
NotI

FIG. 10

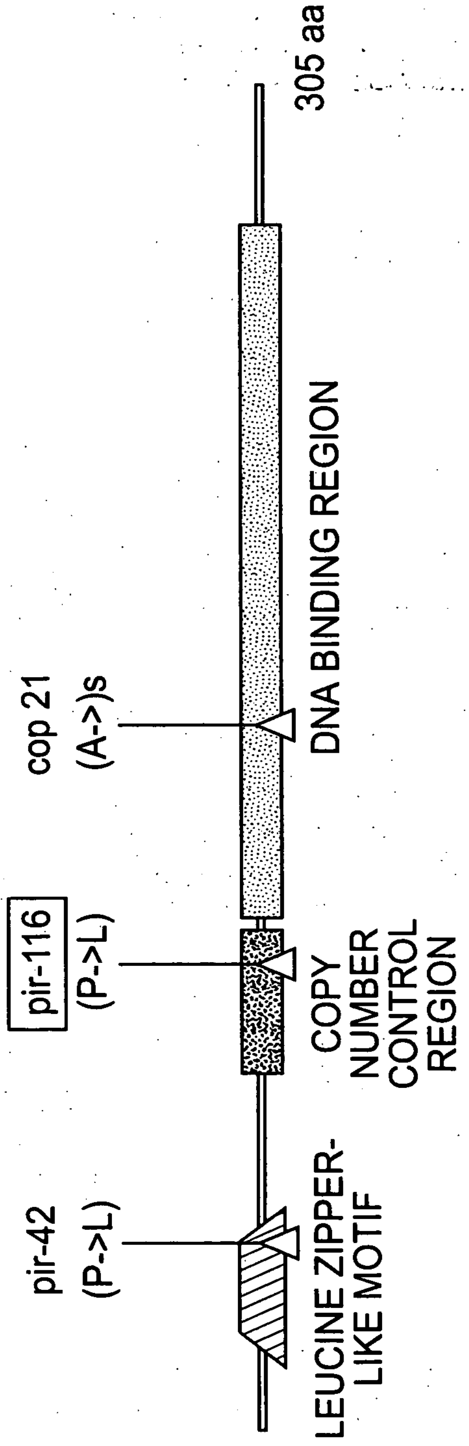

pir-42
(P->L)
LEUCINE ZIPPER-
LIKE MOTIF
pir-116
(P->L)
COPY
NUMBER
CONTROL
REGION
cop 21
(A->)s
DNA BINDING REGION
305 aa

FIG. 11

(SEQ ID NO: 21)

Met Arg Leu Lys Val Met Met Asp Val Asn Lys Lys Thr Lys Ile Arg His Arg
1 ATG AGA CTC AAG GTC ATG ATG GAC GTG AAC AAA AAA ACG AAA ATT CGC CAC CGA

Asn Glu Leu Asn His Thr Leu Ala Gln Leu Pro Leu Pro Ala Lys Arg Val Met
55 AAC GAG CTA AAT CAC ACC CTG GCT CAA CTT CCT TTG CCC GCA AAG CGA GTG ATG

Tyr Met Ala Leu Ala Leu Ile Asp Ser Lys Glu Pro Leu Glu Arg Gly Arg Val
109 TAT ATG CGC CTT CGT CTC ATC GAT AGC AAA GAA CCT CTT GAA CGA GGG CGA GTT

Phe Lys Ile Arg Ala Glu Asp Leu Ala Ala Leu Ala Lys Ile Thr Pro Ser Leu
163 TTC AAA ATT AGG GCT GAA GAC CTT GCA GCG CTC GCC AAA ATC ACC CCA TCG CTT

Ala Tyr Arg Gln Leu Lys Glu Gly Gly Lys Leu Lue Gly Ala Ser Lys Ile Ser
217 GCT TAT CGA CAA TTA AAA GAG GGT GGT AAA TTA CTT GGT GCC AGC AAA ATT TCG

Leu Arg Gly Asp Asp Ile Ile Ala Leu Ala Lys Glu Leu Asn Leu Leu Phe Thr
271 CTA AGA GGG GAT GAT ATC ATT GCT TTA GCT AAA GAG CTT AAC CTG CTC TTT ACT

Ala Lys Asn Ser Pro Glu Glu Leu Asp Leu Asn Ile Ile Glu Trp Ile Ala Tyr
325 GCT AAA AAC TCC CCT GAA GAG TTA GAT CTT AAC ATT ATT GAG TGG ATA GCT TAT

Ser Asn Asp Glu Gly Tyr Leu Ser Leu Lys Phe Thr Arg Thr Ile Glu Pro Tyr
379 TCA AAT GAT GAA GGA TAC TTG TCT TTA AAA TTC ACC AGA ACC ATA GAA CCA TAT

Ile Ser Ser Leu Ile Gly Lys Lys Asn Lys Phe Thr Thr Gln Leu Leu Thr Ala
433 ATC TCT AGC CTT ATT GGG AAA AAA AAT AAA TTC ACA ACG CAA TTG TTA ACG GCA

Ser Leu Arg Leu Ser Ser Gln Tyr Ser Ser Ser Leu Tyr Gln Leu Ile Arg Lys
487 AGC TTA CGC TTA AGT AGC CAG TAT TCA TCT TCT CTT TAT CAA CTT ATC AGG AAG

His Tyr Ser Asn Phe Lys Lys Lys Asn Tyr Phe Ile Ile Ser Val Asp Glu Leu
541 CAT TAC TCT AAT TTT AAG AAG AAA AAT TAT TTT ATT ATT TCC GTT GAT GAG TTA

FIG. 12.1

```
    Lys Glu Glu Leu Ile Als Yyr Thr Phe Asp Lys Asp Gly Asn Ile Glu Tyr Lys
595 AAG GAA GAG TTA ATA GCT TAT ACT TTT GAT AAA GAT GGA AAT ATT GAG TAC AAA

    Tyr Pro Asp Phe Pro Ile Phe Lys Arg Asp Val Leu Asn Lys Ala Ile Ala Glu
649 TAC CCT GAC TTT CCT ATT TTT AAA AGG GAT GTG TTA AAT AAA GCC ATT GCT GAA

    Ile Lys Lys Lys Thr Glu Ile Ser Phe Val Gly Phe Thr Val His Glu Lys Glu
703 ATT AAA AAG AAA ACA GAA ATA TCG TTT GTT GGC TTC ACT GTT CAT GAA AAA GAA


    Gly Arg Lys Ile Ser Lys Leu Lys Phe Glu Phe Val Val Asp Glu Asp Glu Phe
757 GGA AGA AAA ATT AGT AAG CTG AAG TTC GAA TTT GTC GTT GAT GAA GAT GAA TTT

    Ser Gly Asp Lys Asp Asp Glu Ala Phe Phe Met Asn Leu Ser Glu Ala Asp Ala
811 TCT GGC GAT AAA GAT GAT GAA GCT TTT TTT ATG AAT TTA TCT GAA GCT GAT GCA

    Ala Phe Leu Lys Val Phe Asp Glu Thr Val Pro Pro Lys Lys Ala Lys Gly ***
865 GCT TTT CTC AAG GTA TTT GAT GAA ACC GTA CCT CCC AAA AAA GCT AAG GGG TGA
```

FIG. 12.2

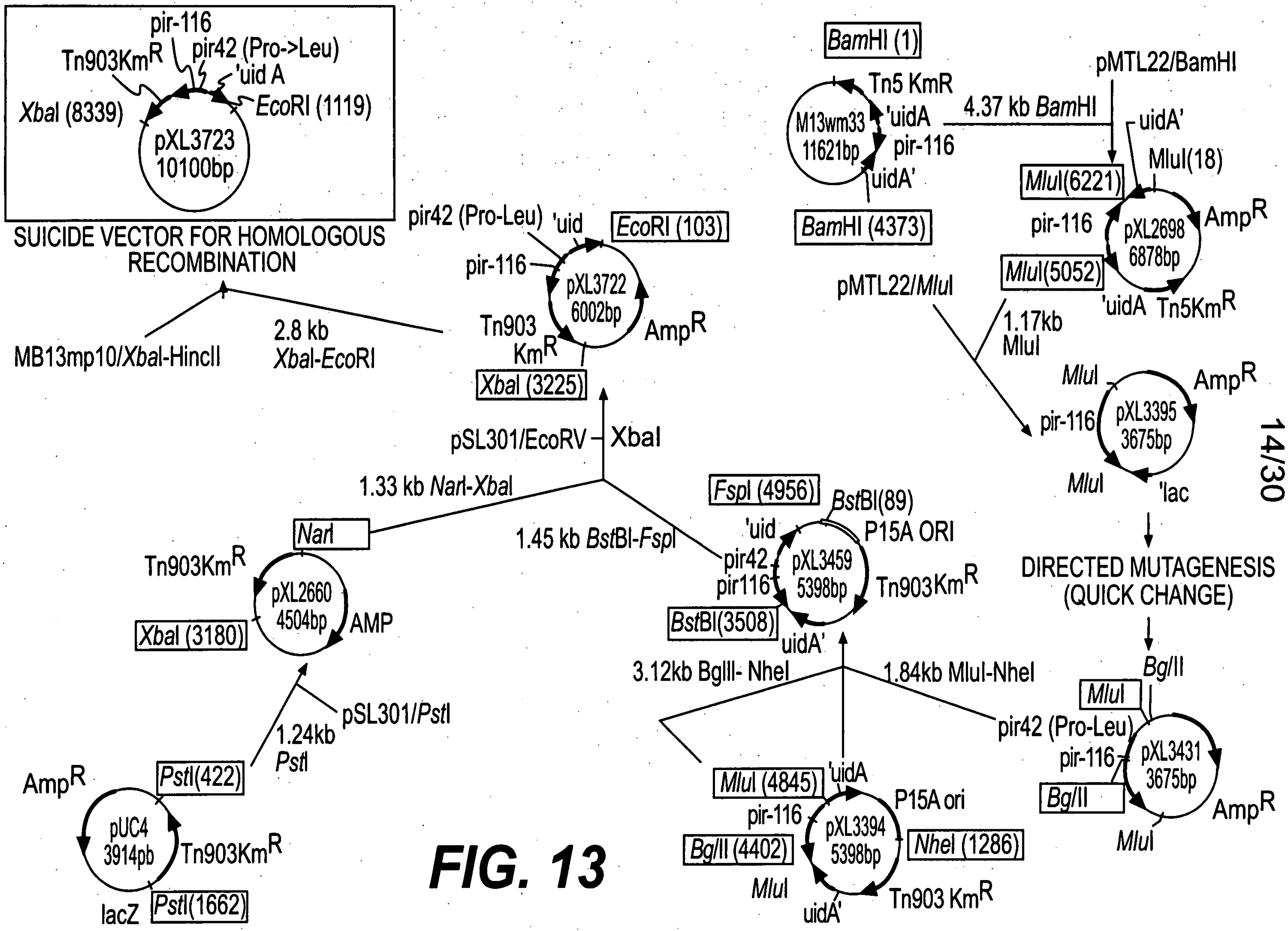
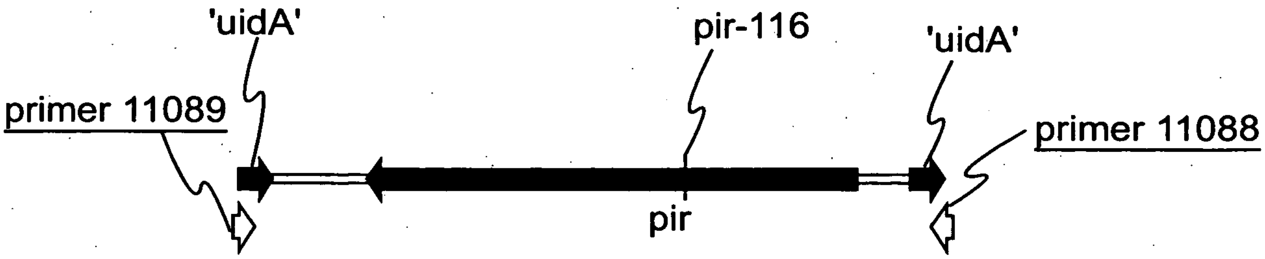
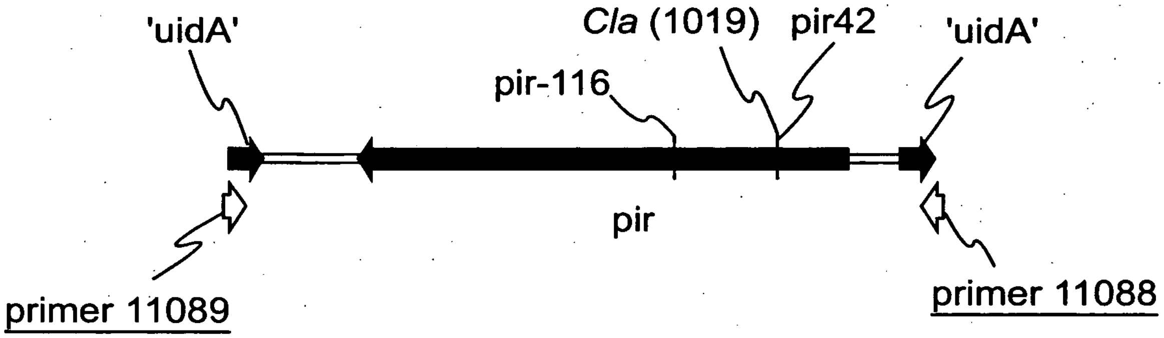

pir-116
Tn903KmR
pir42 (Pro->Leu)
'uid A
XbaI (8339)
EcoRI (1119)
pXL3723
10100bp
SUICIDE VECTOR FOR HOMOLOGOUS RECOMBINATION
MB13mp10/XbaI-HincII
2.8 kb
XbaI-EcoRI
pir42 (Pro-Leu)
'uid
EcoRI (103)
pir-116
pXL3722
6002bp
Tn903
KmR
AmpR
XbaI (3225)
pSL301/EcoRV
XbaI
1.33 kb NarI-XbaI
1.45 kb BstBI-FspI
NarI
Tn903KmR
pXL2660
4504bp
AMP
XbaI (3180)
1.24kb
PstI
pSL301/PstI
AmpR
PstI(422)
pUC4
3914pb
Tn903KmR
lacZ
PstI(1662)
BamHI (1)
Tn5 KmR
M13wm33
11621bp
'uidA
pir-116
uidA'
BamHI (4373)
4.37 kb BamHI
pMTL22/BamHI
uidA'
MluI(18)
MluI(6221)
pir-116
pXL2698
6878bp
AmpR
MluI(5052)
'uidA
Tn5KmR
pMTL22/MluI
1.17kb
MluI
MluI
AmpR
pir-116
pXL3395
3675bp
MluI
'lac
FspI (4956)
BstBI(89)
'uid
P15A ORI
pir42
pir116
pXL3459
5398bp
Tn903KmR
BstBI(3508)
uidA'
DIRECTED MUTAGENESIS
(QUICK CHANGE)
BglI
MluI
pir42 (Pro-Leu)
pir-116
pXL3431
3675bp
BglI
AmpR
MluI
3.12kb BglII- NheI
1.84kb MluI-NheI
MluI (4845)
'uidA
P15A ori
pir-116
pXL3394
5398bp
BglII (4402)
NheI (1286)
MluI
uidA'
Tn903 KmR

FIG. 13



PCR pir116 (11089-11088)
1304 bp



PCR pir116-pir42 (11089-11088)
1304 bp

FIG. 14

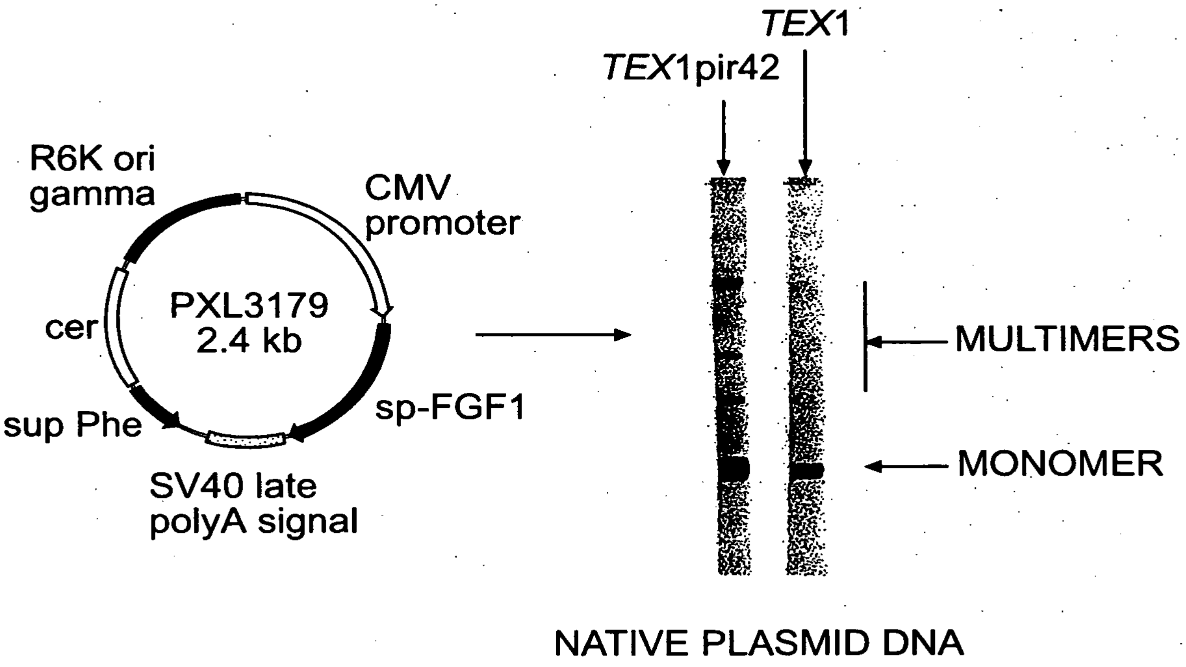

R6K ori gamma
CMV promoter
PXL3179
2.4 kb
cer
sup Phe
sp-FGF1
SV40 late polyA signal
*TEX*1
*TEX*1pir42
MULTIMERS
MONOMER
NATIVE PLASMID DNA

FIG. 15

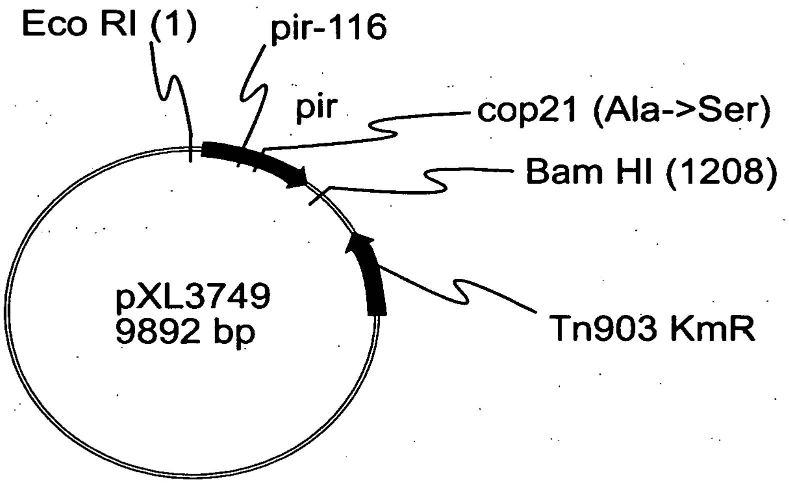
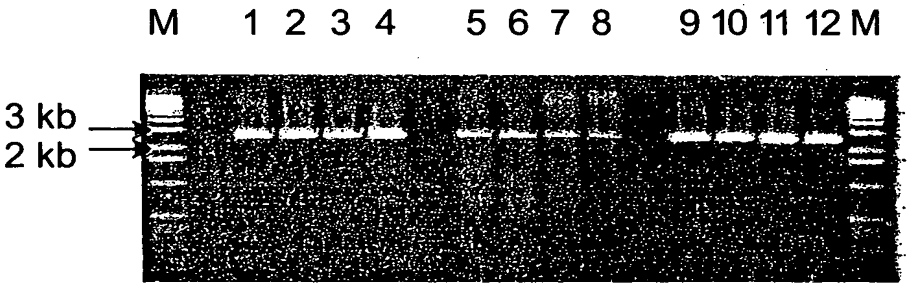

Eco RI (1)
pir-116
pir
cop21 (Ala->Ser)
Bam HI (1208)
Tn903 KmR
pXL3749
9892 bp

FIG. 16



M: 1 kb LADDER (LIFE BIOTECHNOLOGIES)
1->4: TEX1*cop*21 (pXL2979)
5->8: XAC1*pir* (pXL2979)
9->12: TEX1 (pXL2979)

FIG. 17

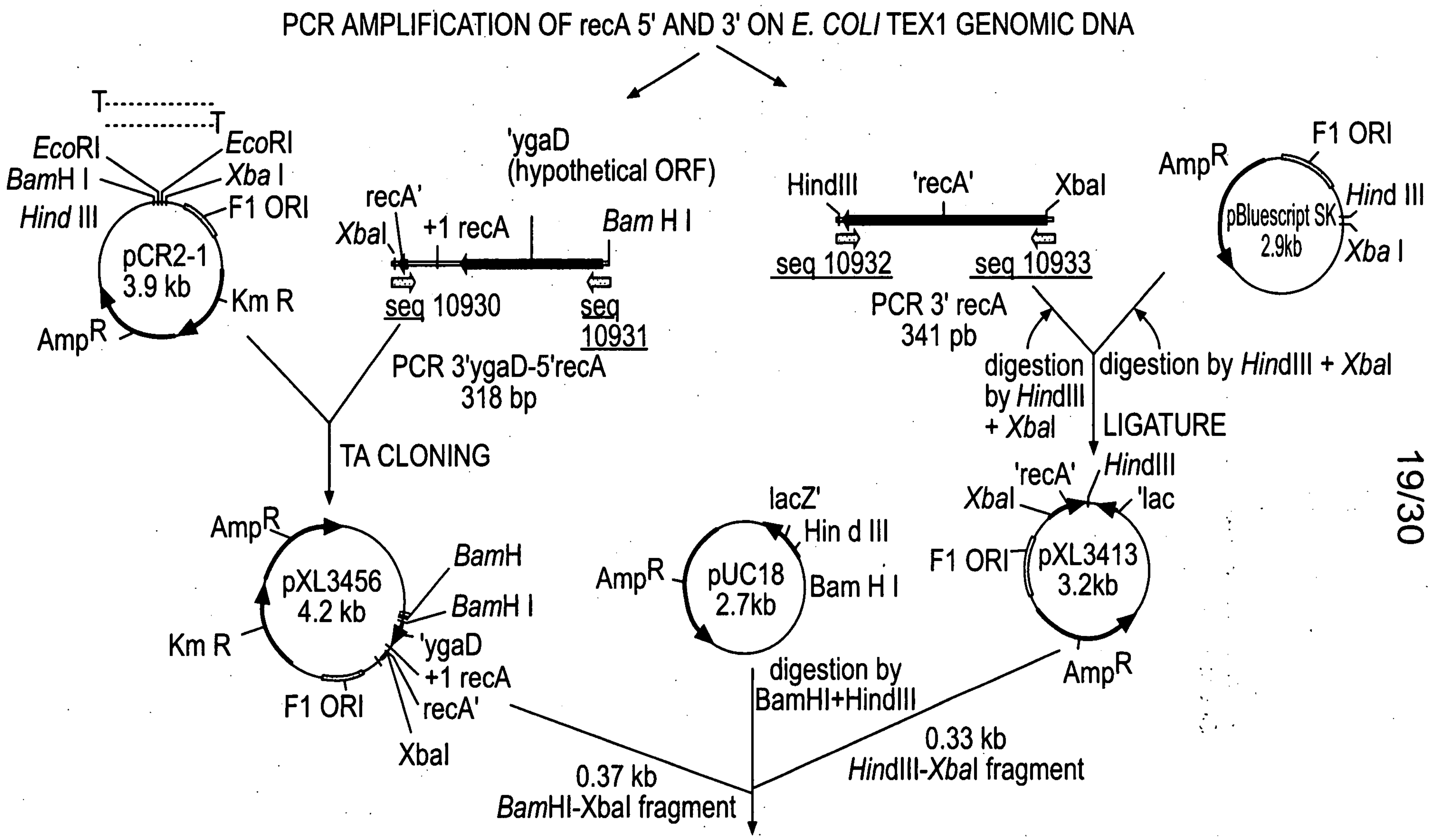
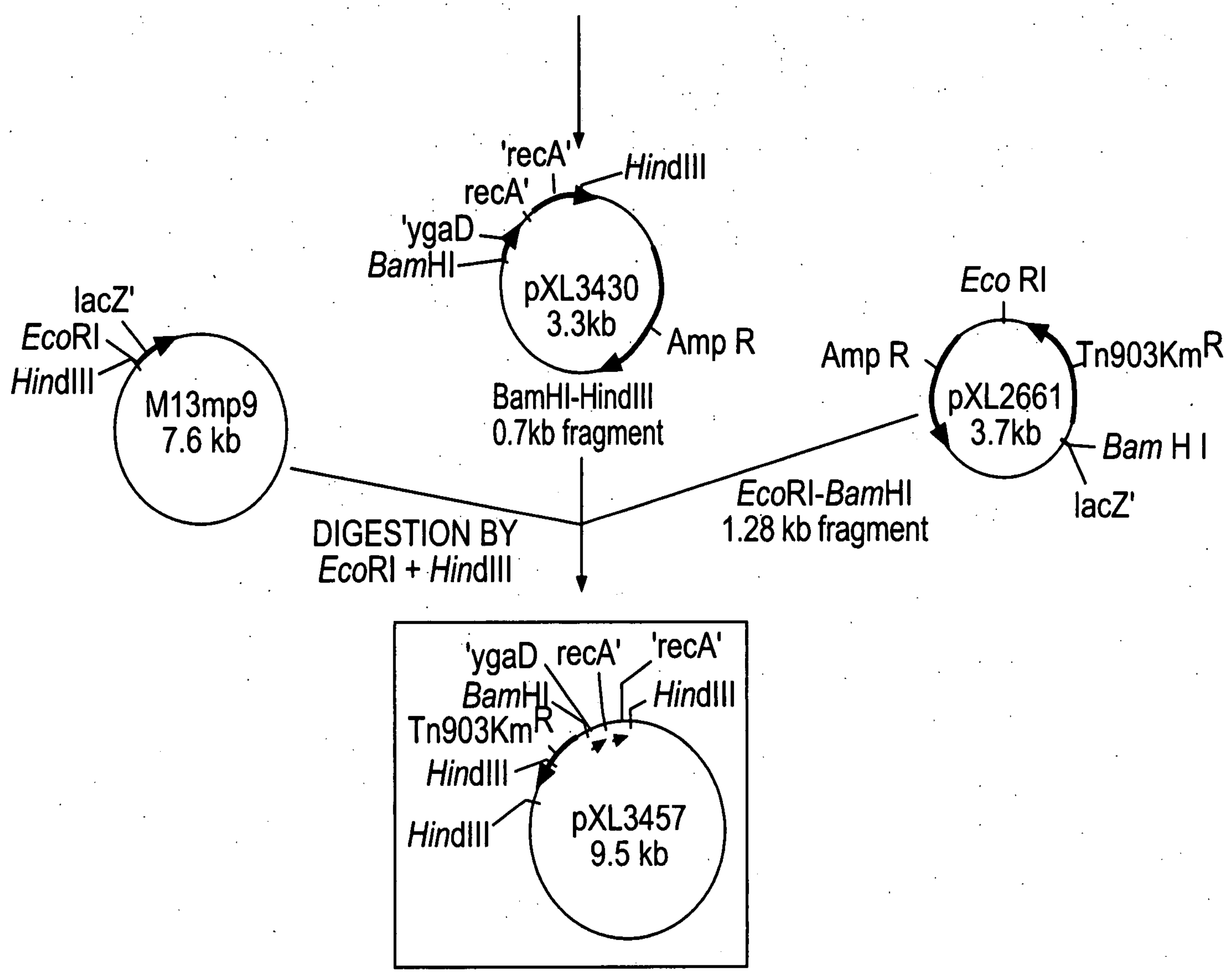
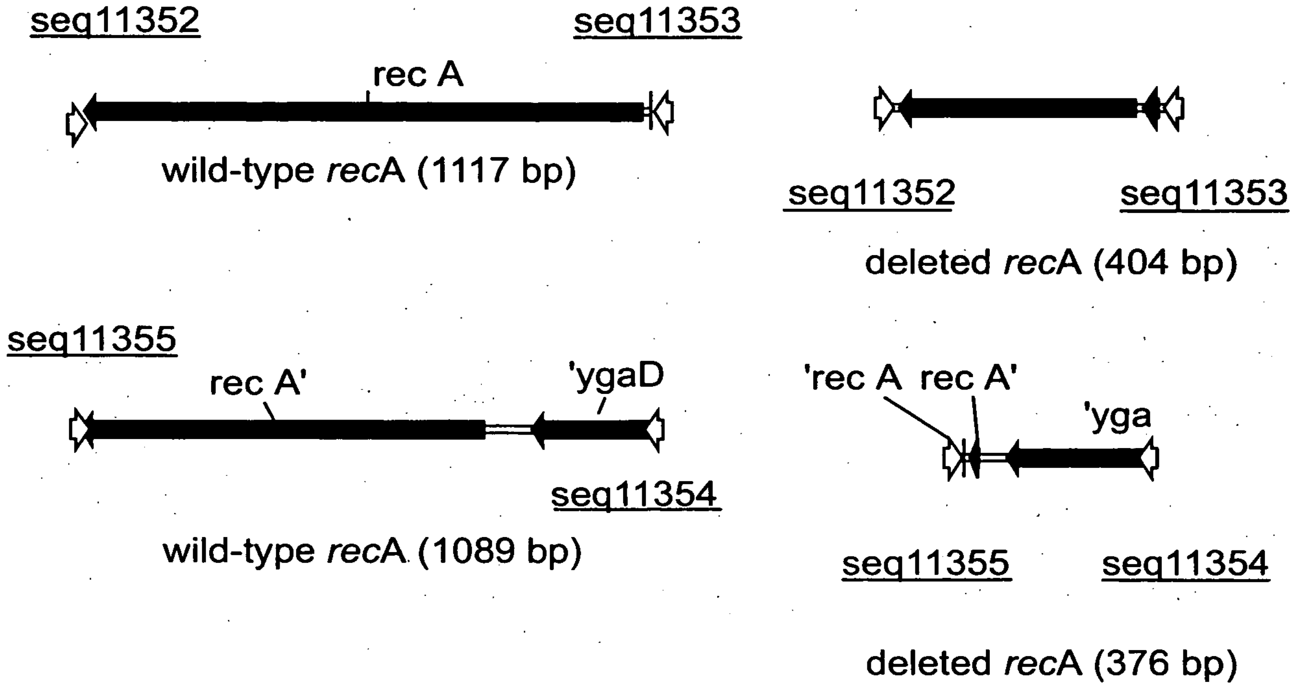

PCR AMPLIFICATION OF recA 5' AND 3' ON *E. COLI* TEX1 GENOMIC DNA
T
T
*Eco*RI
*Bam*H I
Hind III
*Eco*RI
Xba I
F1 ORI
pCR2-1
3.9 kb
Km R
Amp^R
'ygaD
(hypothetical ORF)
recA'
*Xba*I
+1 recA
Bam H I
seq 10930
seq 10931
PCR 3'ygaD-5'recA
318 bp
TA CLONING
HindIII
'recA'
XbaI
seq 10932
seq 10933
PCR 3' recA
341 pb
Amp^R
F1 ORI
pBluescript SK
2.9kb
Hind III
Xba I
digestion by *Hind*III + *Xba*I
digestion by *Hind*III + *Xba*I
LIGATURE
Amp^R
pXL3456
4.2 kb
Km R
F1 ORI
*Bam*H
*Bam*H I
'ygaD
+1 recA
recA'
XbaI
lacZ'
Hin d III
Bam H I
Amp^R
pUC18
2.7kb
digestion by BamHI+HindIII
'recA'
*Hind*III
*Xba*I
'lac
F1 ORI
pXL3413
3.2kb
Amp^R
0.37 kb
*Bam*HI-XbaI fragment
0.33 kb
*Hind*III-*Xba*I fragment

FIG. 18.1



SUICIDE VECTOR FOR RECOMBINATION

FIG. 18.2



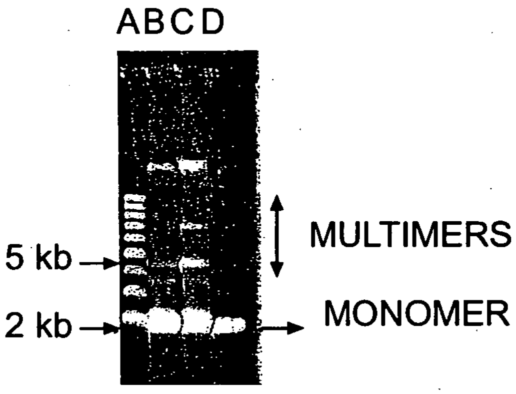
wild-type *rec*A (1117 bp)

deleted *rec*A (404 bp)

wild-type *rec*A (1089 bp)

deleted *rec*A (376 bp)

FIG. 19



A: SUPERCOILED DNA LADDER (PROMEGA)
B: pXL3179 IN TEX2 *pir*42
C: pXL3179 IN TEX1 *pir*42
D: pXL3179 IN TEX1

FIG. 20

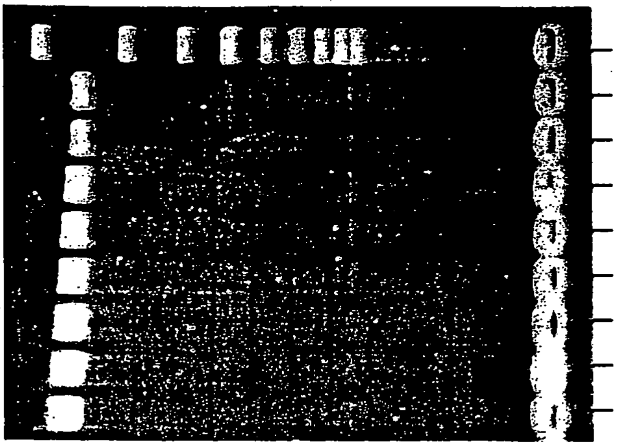
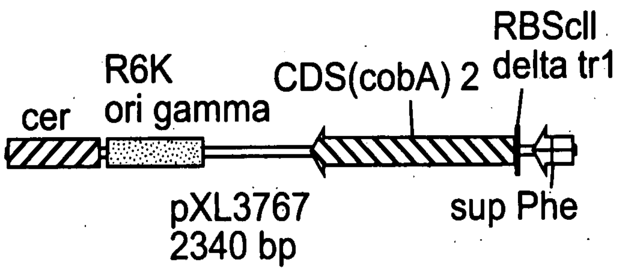

SUPERCOILED DNA LADDER (2,3 . . . Kb)
Op1328S5 18h51
Op1328S5 18h51
Op1328S5 25h30
Op1328S5 25h30
Op1328S5 43h
Op1328S5 43h
Op1328S5 48h47 (FINAL)
Op1328S5 48h47 (FINAL)

FIG. 21


cer
R6K
ori gamma
CDS(cobA) 2
RBScll
delta tr1
pXL3767
2340 bp
sup Phe

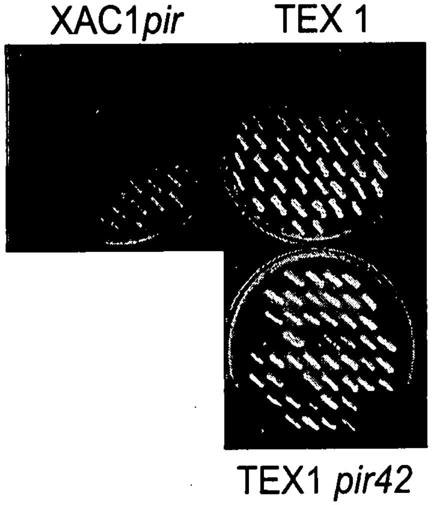

XAC1*pir*
TEX 1
TEX1 *pir42*

FIG. 22

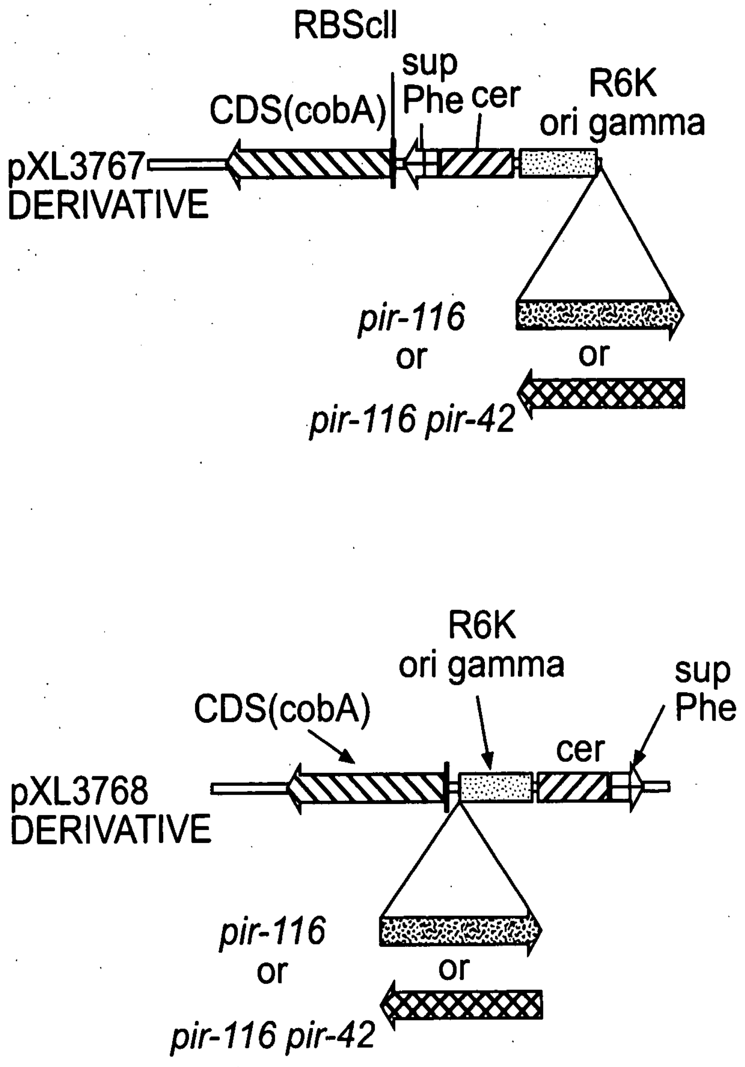

RBScll
sup
Phe
cer
R6K
ori gamma
CDS(cobA)
pXL3767
DERIVATIVE
pir-116
or
pir-116 pir-42
or
R6K
ori gamma
CDS(cobA)
sup
Phe
cer
pXL3768
DERIVATIVE
pir-116
or
pir-116 pir-42
or

FIG. 23

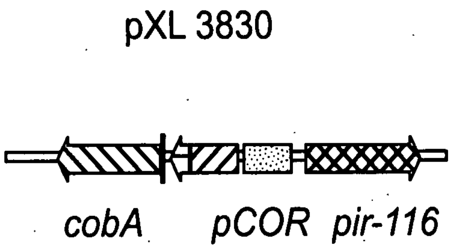

pXL 3830
cobA
pCOR
pir-116

FIG. 24

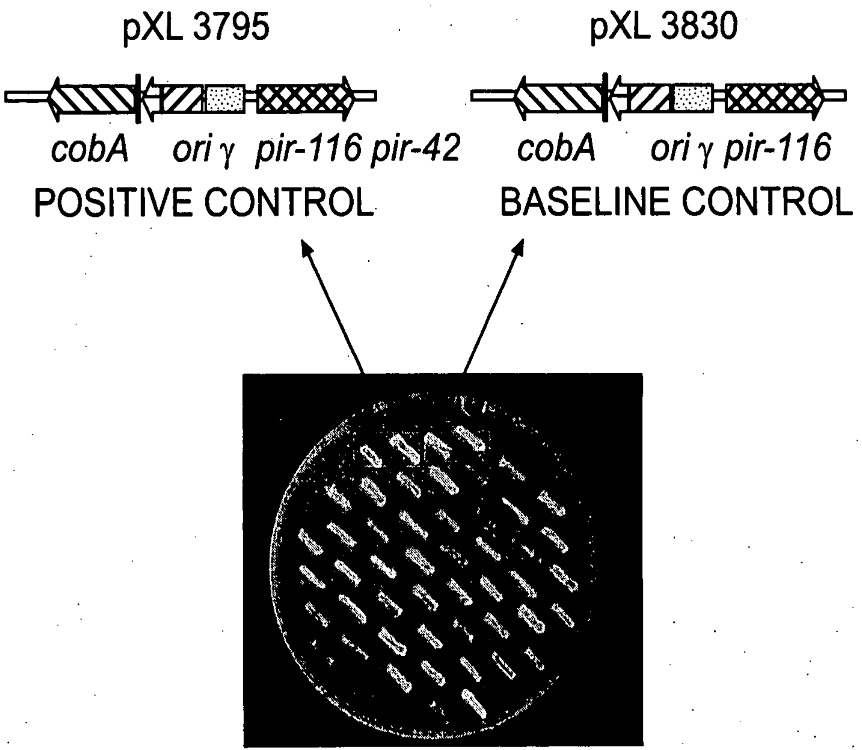

pXL 3795
cobA
ori γ
pir-116 pir-42
POSITIVE CONTROL
pXL 3830
cobA
ori γ
pir-116
BASELINE CONTROL

FIG. 25

PLASMIDS CARRYING	RELATIVE COPY NUMBER	MULTIMERS
pir116	1	**
pir116-pir42	4 TO 6	***
100B	4 TO 5	*
114C	3 TO 5	*
201C	5 TO 9	***

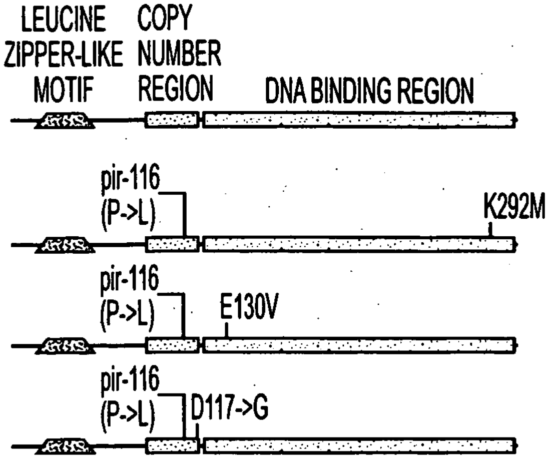


FIG. 26

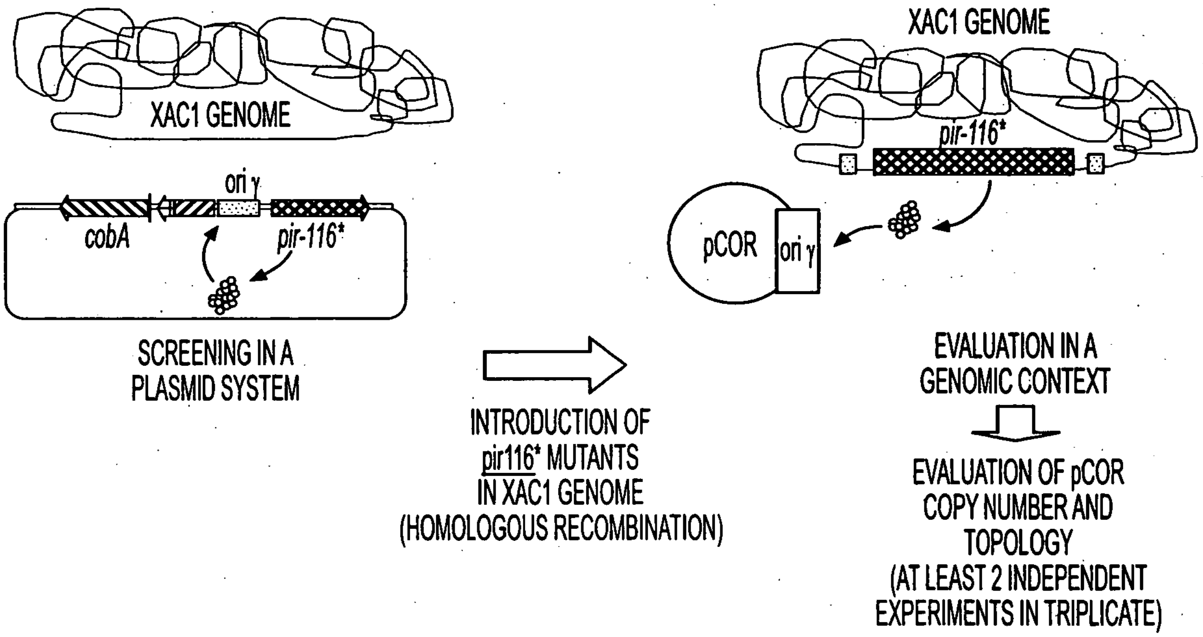

XAC1 GENOME
ori γ
cobA
pir-116*
SCREENING IN A PLASMID SYSTEM
INTRODUCTION OF pir116* MUTANTS IN XAC1 GENOME (HOMOLOGOUS RECOMBINATION)
XAC1 GENOME
pir-116*
pCOR
ori γ
EVALUATION IN A GENOMIC CONTEXT
EVALUATION OF pCOR COPY NUMBER AND TOPOLOGY (AT LEAST 2 INDEPENDENT EXPERIMENTS IN TRIPLICATE)

FIG. 27

pXL3179= NV1FGF (2.4 kb)

E. Coli STRAINS	pXL13179 COPY NUMBER	MULTIMERS
XAC1pir116	1	NONE
TEX1pir42	3 TO 4	**
100B1	1.5 TO 2	NONE
114C2	1.5 TO 2	**
201C	2 TO 3	****

FIG. 28

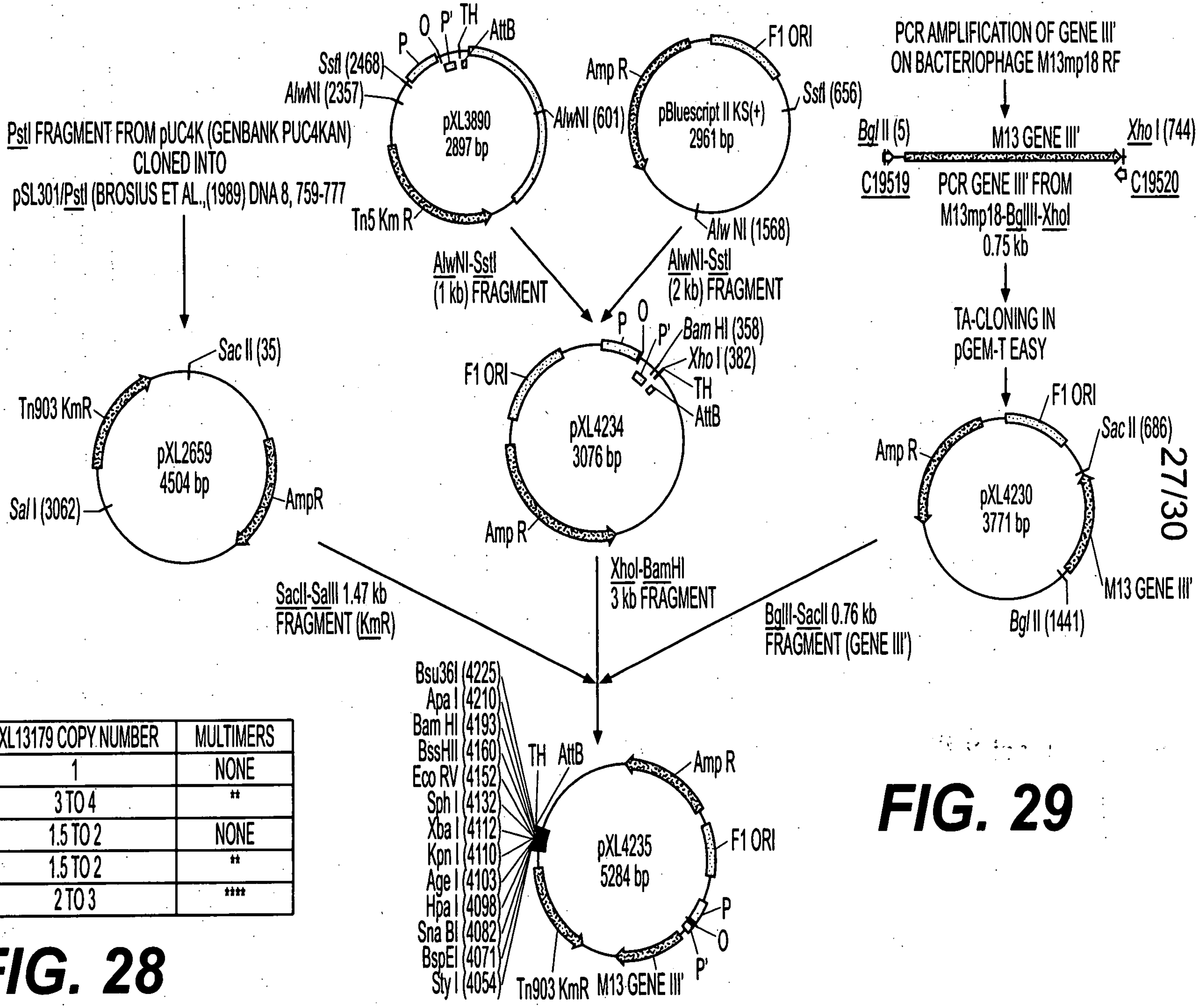


FIG. 29

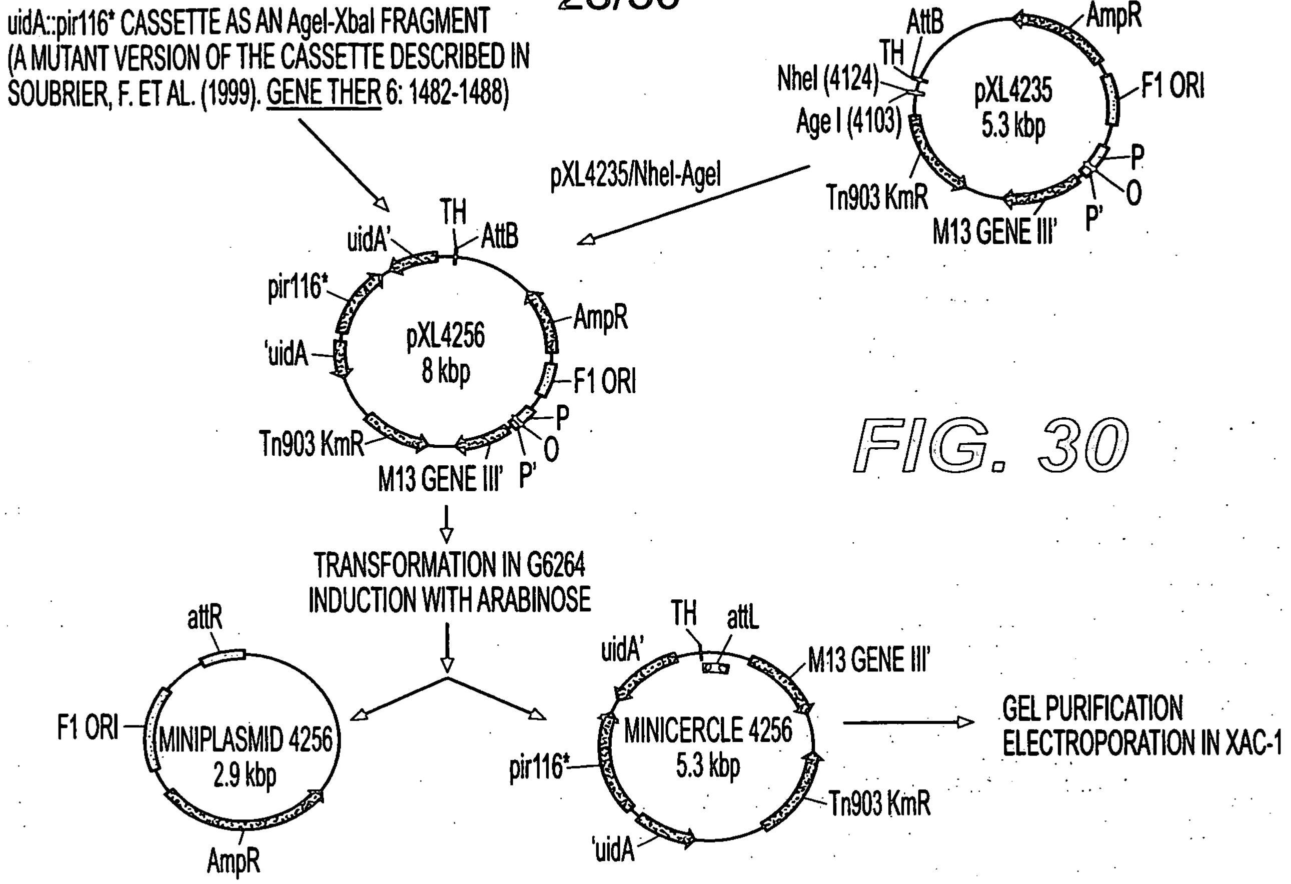

uidA::pir116* CASSETTE AS AN AgeI-XbaI FRAGMENT
(A MUTANT VERSION OF THE CASSETTE DESCRIBED IN
SOUBRIER, F. ET AL. (1999). GENE THER 6: 1482-1488)
AttB
TH
NheI (4124)
Age I (4103)
AmpR
F1 ORI
pXL4235
5.3 kbp
P
O
P'
Tn903 KmR
M13 GENE III'
pXL4235/NheI-AgeI
TH
AttB
uidA'
pir116*
'uidA
pXL4256
8 kbp
AmpR
F1 ORI
P
O
P'
Tn903 KmR
M13 GENE III'
TRANSFORMATION IN G6264
INDUCTION WITH ARABINOSE
attR
F1 ORI
MINIPLASMID 4256
2.9 kbp
AmpR
TH
attL
uidA'
M13 GENE III'
MINICERCLE 4256
5.3 kbp
pir116*
Tn903 KmR
'uidA
GEL PURIFICATION
ELECTROPORATION IN XAC-1

FIG. 30

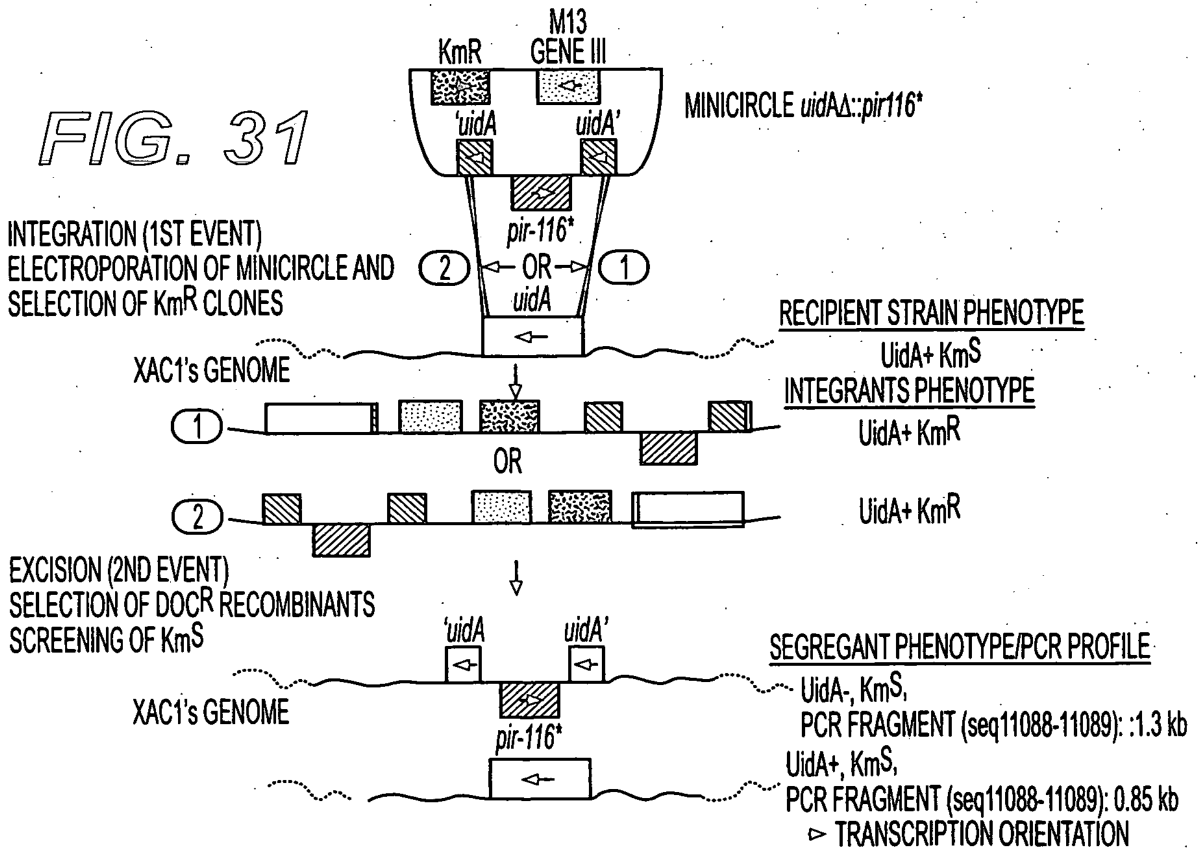

M13
KmR
GENE III
MINICIRCLE uidAΔ::pir116*
'uidA
uidA'
pir-116*
INTEGRATION (1ST EVENT)
ELECTROPORATION OF MINICIRCLE AND
SELECTION OF KmR CLONES
2
OR
1
uidA
RECIPIENT STRAIN PHENOTYPE
UidA+ KmS
XAC1's GENOME
INTEGRANTS PHENOTYPE
1
UidA+ KmR
OR
2
UidA+ KmR
EXCISION (2ND EVENT)
SELECTION OF DOCR RECOMBINANTS
SCREENING OF KmS
'uidA
uidA'
SEGREGANT PHENOTYPE/PCR PROFILE
XAC1's GENOME
UidA-, KmS,
PCR FRAGMENT (seq11088-11089): :1.3 kb
pir-116*
UidA+, KmS,
PCR FRAGMENT (seq11088-11089): 0.85 kb
▻ TRANSCRIPTION ORIENTATION

FIG. 31

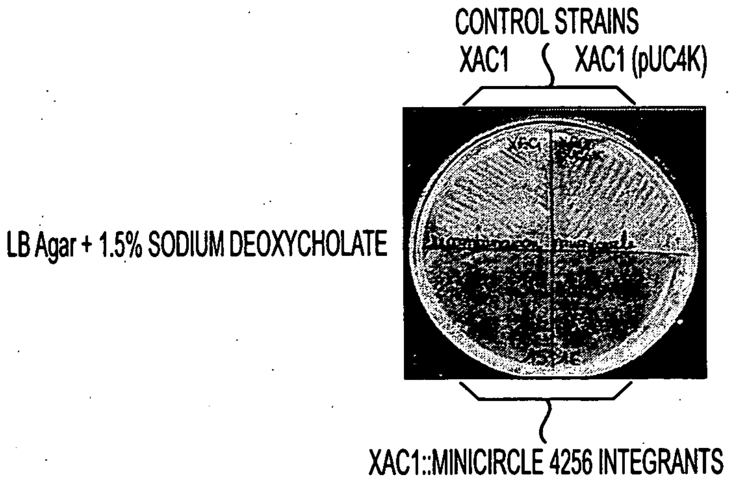

CONTROL STRAINS
XAC1
XAC1 (pUC4K)
LB Agar + 1.5% SODIUM DEOXYCHOLATE
XAC1::MINICIRCLE 4256 INTEGRANTS

FIG. 32

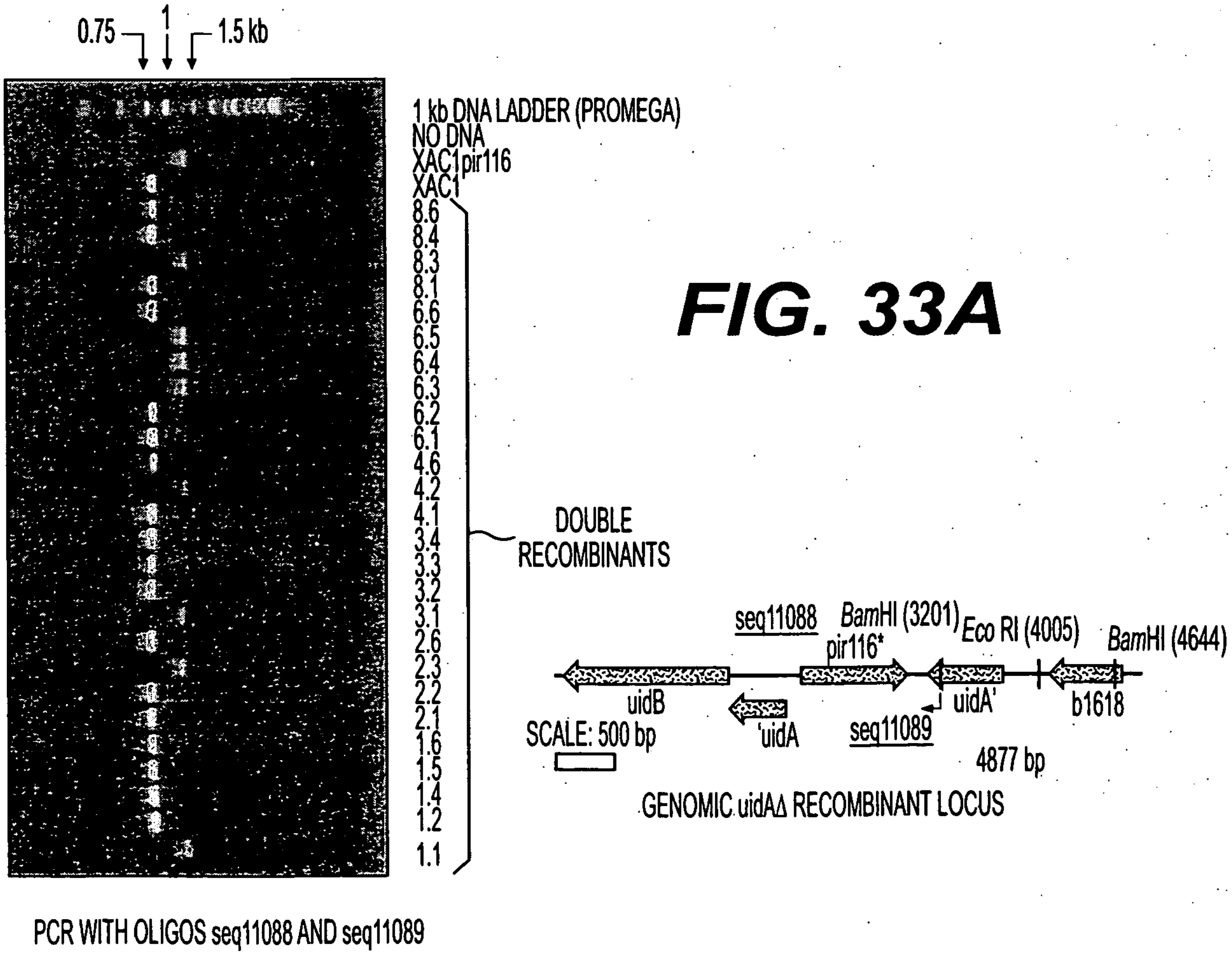
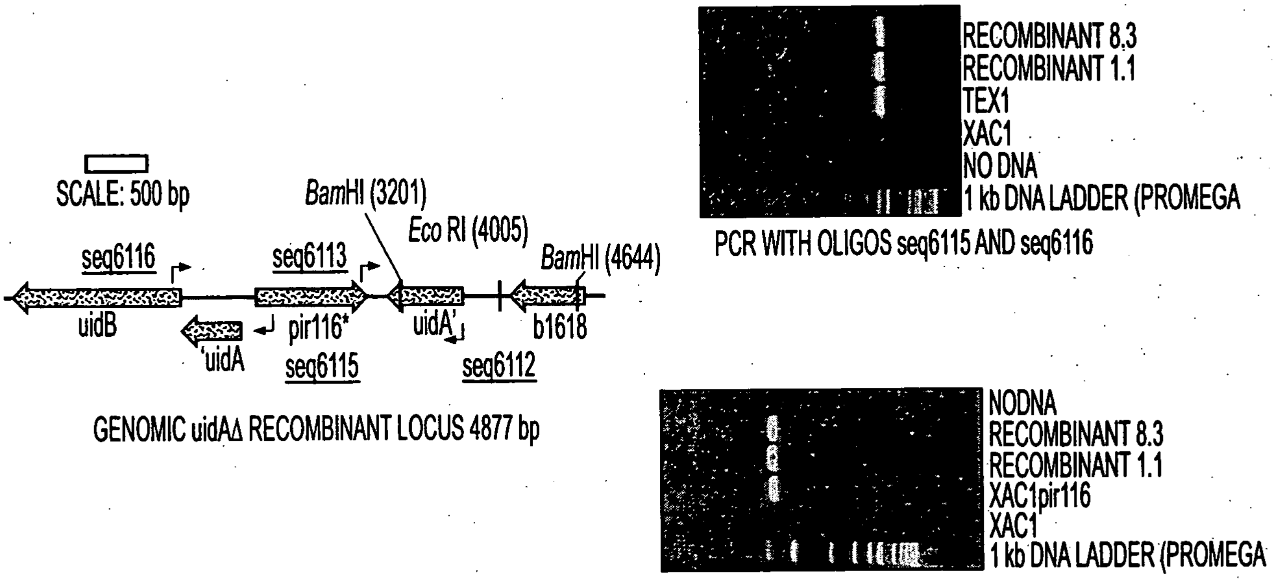


FIG. 33A



GENOMIC uidAΔ RECOMBINANT LOCUS 4877 bp

PCR WITH OLIGOS seq6115 AND seq6116

PCR WITH OLIGOS seq6112 AND seq6113

FIG. 33B